BINDING MOLECULES DERIVED FROM IMMUNOGLOBULINS WHICH DO NOT TRIGGER COMPLEMENT MEDIATED LYSIS

TECHNICAL FIELD

5   The present invention relates to binding polypeptides having amino acid sequences derived from a modified constant region of the immunoglobulin G (IgG) heavy chain. The invention further relates to methods and materials for producing such polypeptides, and methods

10   and materials employing them.

PRIOR ART

Immunoglobulins

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Immunoglobulins are glycoproteins which help to defend the host against infection. They generally consist of heavy and light chains, the N-terminal domains of which form a variable or V domain capable of binding antigen.

20   The V domain is associated with a constant or C-terminal domain which defines the class (and sometimes subclass [isotype], and allotype [isoallotype]) of the immunoglobulin.

25   Thus in mammalian species immunoglobulins exist as IgD, IgG, IgA, IgM and IgE. The IgG class in turn exists as 4 subclasses in humans (IgG1, IgG2, IgG3, IgG4). The C-domain in IgGs comprises three domains $C\gamma1$, $C\gamma2$, and $C\gamma3$, which are very similar between these subclasses (over 90%

30   homology). The $C\gamma1$ and $C\gamma2$ domains are linked by a hinge. The role of the subclasses appears to vary between species.

- 2 -

It is known that the C-domain is responsible for various effector functions of the immunoglobulin (see Clark (1997) "IgG Effector Mechanisms" in "Antibody Engineering" Ed. Capra, Pub. Chem Immunol,

5   Basel, Kurger, Vol 65 pp 88-110, for a detailed review).

Briefly, IgG functions are generally achieved via interaction between the Fc region of the Ig and an Fcγ receptor (FcγR) or other binding molecule, sometimes on

10   an effector cell. This can trigger the effector cells to kill target cells to which the antibodies are bound through their variable (V) regions. Also antibodies directed against soluble antigens might form immune complexes which are targeted to FcγRs which result in the

15   uptake (opsonisation) of the immune complexes or in the triggering of the effector cells and the release of cytokines.

In humans, three classes of FcγR have been characterised,

20   although the situation is further complicated by the occurrence of multiple receptor forms. The three classes are:

(i) FcγRI (CD64) binds monomeric IgG with high affinity

25   and is expressed on macrophages, monocytes, and sometimes neutrophils and eosinophils.

(ii) FcγRII (CD32) binds complexed IgG with medium to low affinity and is widely expressed. These receptors can be

30   divided into two important types, FcγRIIa and FcγRIIb.

The 'a' form of the receptor is found on many cells involved in killing (e.g. macrophages, monocytes,

neutrophils) and seems able to activate the killing process, and occurs as two alternative alleles.

The 'b' form seems to play a role in inhibitory processes and is found on B-cells, macrophages and on mast cells and eosinophils. On B-cells it seems to function to suppress further immunoglobulin production and isotype switching to say for example the IgE class. On macrophages, the b form acts to inhibit phagocytosis as mediated through FcγRIIa. On eosinophils and mast cells the b form may help to suppress activation of these cells through IgE binding to its separate receptor.

(iii) FcγRIII (CD16) binds IgG with medium to low affinity and exists as two types. FcγRIIIa is found on NK cells, macrophages, eosinophils and some monocytes and T cells and mediates ADCC. FcγRIIIb is highly expressed on neutrophils. Both types have different allotypic forms.

As well as binding to FcγRs, IgG antibodies can activate complement and this can also result in cell lysis, opsonisation or in cytokine release and inflammation. The Fc region also mediates such properties as the transportation of IgGs to the neonate (via the so-called 'FcRn'); increased half-life (also believed to be effected via an FcRn-type receptor - see Ghetie and Ward (1997) Immunology Today 18, 592-598) and self-aggregation. The Fc-region is also responsible for the interaction with protein A and protein G (which interaction appears to be analogous to the binding of FcRn).

Engineering immunoglobulins

- 4 -

Many of the Fc-mediated properties discussed above may be desirable in naturally occurring or artificially constructed antibodies. However, there are circumstances where, in particular, the cell killing, or the cytokine release and resulting inflammation, is inappropriate and undesirable.

Equally, however, it may be desirable to retain certain Fc-mediated functions, for instance the long plasma half life.

It is known that human IgG4, for example, does not activate complement and human IgG2 does not bind to the high affinity FcγRI receptor and so these have previously been used in some situations (TNF receptor fusion protein was made with IgG4 Fc).

However no human subclass lacks all of the relevant Fc effector triggering functions or complement activation in all circumstances, possibly owing to the existence of the several forms of the FcγRs. Thus, for instance, IgG4 can trigger antibody dependent cellular cytotoxicity (ADCC) in some people and IgG2 binds to one allelic form of the FcγRIIa receptor and also activates complement.

An alternative approach has been to mutate the Fc sequence to substitute residues crucial for function. Certain target residues have been identified and published (see review by Clark 1997, supra). These include the N-linked carbohydrate attached to the conserved site in the C_H2 domain, certain residues in the lower hinge region (eg the sequence ELLGGP) and a proline residue at position 331 and a sequence E-x-K-x-K at

positions 318-322. One recent example is disclosed by
Cole et al (1997) Journal of Immunology 159, 3613-3621.
In that disclosure residues 234, 235 and 237 were mutated
to Alanines (or in the case of 235, sometimes to Glu).
However these are all unusual residues at these positions
in human IgG, thus the presence of such inappropriate
amino acids may make the Fc more immunogenic or antigenic
and may also lead to the loss of certain desirable Fc
functions.

Again this strategy has been used for the construction of
a therapeutic aglycosylated CD3 antibody (see Routledge
et al, 1993 Eur J Immunol 23: 403-411; see also UK PA
9206422.9) and for an inhibitory CD18 antibody. However
one disadvantage here is that the new recombinant
constructs have unusual sequences and may be recognised
and rejected by the immune system as foreign.
Aglycosylated antibodies also lack binding to the
inhibitory receptor FcγRIIb, whereas maintaining this
binding may be advantageous for some applications.

Other approaches to modifying immunoglobulins are
disclosed in WO 92/16562 (Lynxvale Ltd) which discusses
modifying the allotype of the humanised IgG1 antibody
CAMPATH1H which has binding affinity for antigen CD52.
The CD52 antigen is found on human lymphocytes and
monocytes and has been used as a therapeutic target for
treatment of T and B-cell lymphomas and leukeamias,
immunosuppresion of organ and bone-marrow transplant
recipients and also treatment of some autoimmune and
related disorders such as rheumatoid arthritis and
systemic vasculitis.

WO 95/05468 (Lynxvale Ltd) also disclosed the modification of allotypic determinants in Igs (or derivatives) having desired binding or other effector functions.

It can be seen from the forgoing that the provision of methods or materials which would facilitate the engineering of Fc regions such as to reduce unwanted effects, while retaining or enhancing desirable properties, would provide a contribution to the art.

DISCLOSURE OF THE INVENTION

The present inventors have used novel combinations of human IgG subclass sequences to generate chimaeric polypeptides comprising non-natural, human-mimicing Fc sequences which nevertheless do not activate complement or trigger cytotoxic activities through $Fc\gamma R$. At the same time certain desirable IgG properties have been retained. For instance the polypeptides do not contain 'non-human' amino acids, and are therefore likely to have reduced immunogenicity. Further, they still bind Protein A, which is consistent with being able to cross the human placenta through interaction with FcRn (neonatal Fc receptor).

The manner by which the sequences were developed, and certain demonstrated properties, will be discussed in more detail hereinafter. However, briefly, the inventors formulated numerous constructs based on three different IgG sequences (1, 2 and 4). Although the relevant regions of these antibodies share homology, they do not precisely correspond in terms of length, thereby

complicating the process of generating derivative
sequences which retain activities from the natural
sequences. The constructed antibodies were compared with
the parental control antibodies in the context of model
antigen systems RhD (Fog1) and CD52 (CAMPATH-1H).
Surprisingly, a number of sequences were developed with
the required combination of activities not found in the
parent molecules. Generally speaking these contained 1
or more regions or blocks which contained a modification
(generally 2, 3 or 4 amino acids) which was in conformity
with the corresponding region from a different subclass.
Two particular regions or blocks of interest were 233-236
and 327,330,331.

Thus in a first aspect of the present invention there is
disclosed a polypeptide binding molecule comprising (i) a
binding domain capable of binding a target molecule, and
(ii) an effector domain having an amino acid sequence
substantially homologous to all or part of a constant
domain of a human immunoglobulin heavy chain;
characterised in that the binding molecule is capable of
binding the target molecule without triggering
significant complement dependent lysis, or cell mediated
destruction of the target, and preferably whereby the
effector domain is capable of specifically binding FcRn
or FcγRIIb, more preferably both FcRn and FcγRIIb.

The specific binding of FcRn may be evidenced by the
capability to specifically bind protein A.

Thus the binding molecules according to the present
invention have improved clinical properties (e.g. in the
context of 'blocking' antibodies). This is achieved by

- 8 -

the provision of an Fc-derived effector domain which has
a reduced affinity for FcγRI, FcγRIIa and FcγRIII, but
which retains the ability to bind protein A (and hence

FcRn, hence permitting neonatal transport and high half

5   life) and/or FcγRIIb. Thus the residues responsible for
binding FcRn in IgGs need not be modified with respect to
a natural Fc region in the molecules of the present
invention.

10   Generally the reduction in affinity which the effector
region has for the receptor FcγRI (as compared with one
Fc region from which it is derived) may, in preferred
embodiments, be of the order of 100 fold or more. For
certain of the lower affinity receptors discussed above

15   the reduction in affinity may be less e.g. around 2-10
fold, although in the most preferred embodiments it could
be as high 500 fold. Generally the corresponding
reduction in activity in the chemiluminescence assay (as
described in more detail below) may be as high as 30-300

20   fold. The reduced complement activity may be of the
order of 50 fold. The corresponding figure for ADCC may
be much higher e.g. 10,000 fold. However those skilled
in the art will appreciate that the combination of these
(reduced) activities may still be of benefit in certain

25   applications, regardless of the precise level of
reduction.

Although IgG1/IgG2 and IgG1/IgG4 chimeras have been
prepared in the past (see e.g. Morgan et al (1995)

30   Immunology 86: 319-324, or Chappel et al (1991) Proc Natl
Acad Sci USA 88: 9036-9040, or Greenwood et al (1993) Eur
J Immunol 23: 1098-1104) none of these has been shown to
have the combination of properties possessed by the

binding molecules of the present invention.

The various functions of the binding molecule can be assessed without burden by those skilled in the art, for instance by using methods as disclosed below, or methods analogous to these. For instance, the FcγR binding properties may be assessed directly, or indirectly e.g. through inability to trigger monocyte chemiluminescence.

Specifically, the inability to trigger significant complement dependent lysis (which will generally be through a reduced affinity for the C1q molecule) can be measured by CR-51 release from target cells in the presence of the complement components e.g. in the form of serum (as described below) whereby the binding molecule causes less than 5%, preferably less than 2% specific target cell lysis.

Similarly, cell mediated destruction of the target may be assessed by CR-51 release from target cells in the presence of suitable cytotoxic cells e.g. blood mononuclear effector cells (as described below) whereby the binding molecule causes less than 5%, preferably less than 2% target cell lysis.

As an alternative to direct measurement, functionality may be inferred by the ability to inhibit these attributes in functional immunoglobulins. For instance by providing a protective effect against the complement lysis of cells, or the killing of cells (e.g. by ADCC), or by inhibiting the response of monocytes to sensitised cells.

In one, preferred, embodiment of this aspect of the
invention the effector domain comprises an amino acid
sequence substantially homologous to the C_H2 sequence from
human IgG1, G2 or G4, said sequence comprising one or
more of the following modifications (amino acid
substitutions or deletions) at the stated positions,
numbered with respect to the EU numbering system (see
Kabat et al "Sequences of proteins of immunological
interest". Bethesda, US Department of Health and Human
Services, NIH, 1991):

Posn	Amino acid
233	P
234	V
235	A
236	(No residue) or G
327	G
330	S
331	S

In a preferred embodiment, these substitutions are made
in 'blocks' of 233-236 and/or 327,330,331. Thus the
mutated region in the C_H2 domain will be 100% homologous
to the subclass from which the substituted residues
originated, thereby reducing the likelihood that the
region will represent a B-cell or T-cell epitope for the
immune system.

Several mutant immunoglobulins based on IgG1, IgG2, or
IgG4 having the stated features, have been prepared and
have shown to have the required properties. Although
some of the individual residue mutations have been
prepared in binding molecules of the prior art, the

- 11 -

specified combinations are novel as are the achieved functionalities.

Preferred forms of the binding molecule will now be discussed in more detail:

The effector domain

The peptide comprises an effector domain having an amino acid sequence substantially homologous to all or part of a human immunoglobulin constant region, preferably an IgG C-domain.

Numerous sequences for human C regions have been published; see e.g. Clark (1997) supra. Other sequences for human immunoglobulin heavy chains can be obtained from the SwissProt and PIR databases using Lasergene software (DNAStar Limited, London UK) under accession numbers A93433, B90563, A90564, B91668, A91723 and A02146 for human Igγ-1 chain C region, A93906, A92809, A90752, A93132, A02148 for human Igγ-2 chain C region, A90933, A90249, A02150 for human Igγ-4 chain C region, and A23511 for human Igγ-3 chain C region.

Homology (or identity, or similarity) may be assessed by any convenient method. Homology may be at the encoding nucleotide sequence or encoded amino acid sequence level. By "substantially homologous" is meant that the comprised amino acid sequence shares at least about 50%, or 60%, or 70%, or 80% homology, most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% homology with the reference immunoglobulin.

Similarity or homology may be as defined and determined
by the TBLASTN program, of Altschul et al. (1990) J. Mol.
Biol. 215: 403-10, which is in standard use in the art,
or, and this may be preferred, the standard program
BestFit, which is part of the Wisconsin Package, Version
8, September 1994, (Genetics Computer Group, 575 Science
Drive, Madison, Wisconsin, USA, Wisconsin 53711).
BestFit makes an optimal alignment of the best segment of
similarity between two sequences. Optimal alignments are
found by inserting gaps to maximize the number of matches
using the local homology algorithm of Smith and Waterman.

This assessment can be made without burden by a person of
ordinary skill in the art, in conjunction with assessing
the required combination of activities, in order to
recognise a molecule of the present invention.

In addition to having the reduced affinity for FcγRI,
FcγRIIa FcγRIIIa and FcγRIIIb, it may be desirable that
an ability to bind the 'inhibitory' receptor FcγRIIb is
retained or possessed to some degree by the effector
molecule, and preferably is higher than its affinity for
the FcγRIIa receptor, and more preferably commensurate
with that of a parent Ig domain from which it is derived.
Results obtained by the present inventors indicate that
the binding molecules which they have developed do have
this property. Hitherto it was not appreciated in the art
that the binding of Fc regions to FcγRIIa and FcγRIIb
could be manipulated independently. This ability may
complement the other required functions (as indicated by
the ability to bind protein A) in increasing the
therapeutic potential of the binding molecule.

In particular, a number of publications have highlighted the important role that FcγRIIb may play in inhibiting cellular processes (see Daeron et al, 1995 Immunity 3(5): 635-46; Van den Herik et al, 1995 Blood 85(8):2201-11;

5   Sarmay et al, 1996 Immunol Lett 54(2-3): 93-100; Fong et al, 1996 Immunol Lett 54(2-3): 83-91; Sarmay et al, 1996 J Biol Chem 271(48): 30499-504; Unkeless & Jin, 1997 Curr Opin Immunol 9(3): 338-43; Isakov, 1997 Immunol Res 16(1):85-100; Hunter et al, 1998 Blood 91(5): 1762-8;

10   Malbec et al, 1998 J Immunol 160(4): 1647-58; Clynes et al, 1999 J Exp Med 189(1): 179-85). These workers showed that FcγRIIb, when cross-linked to other receptors, could inhibit signalling from them, thereby inhibiting such processes as B cell activation, mast cell degranulation,

15   and phagocytosis by macrophages.

Thus binding molecules of the present invention which retain this activity could be used not only to compete with, and competitively inhibit, undesirable antibody-

20   antigen (such as autoantigens or alloantigens) interactions, but also to non-competitively inhibit these processes e.g. by preventing further autoantibody or alloantibody production by inhibition of B cell activation.   Other example applications for this

25   inhibitory effect are discussed below in relation to allergy and asthma therapeutics (inhibition of mast cell degranulation) and anti-RhD molecules (inhibition of phagocytosis).

30   Preferably the effector domain is itself derived from a human immunoglobulin constant region, more preferably an IgG C-domain.

- 14 -

Preferably the comprised amino acid sequence is substantially homologous to the C_H2 sequence (i.e. approximately residues 231-340) from human IgG1, G2 or G4, having the modified amino acids discussed above.

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The most preferred C_H2 sequences are shown in Fig 17, particularly those designated G1Δab, G2Δa, or G1Δac respectively.

10   Any of these sequences may be combined with (e.g run contiguously with) natural or modified C_H3 and natural or modified hinge region, plus optionally C_H1, sequences in the molecules of the present invention.

15   However it will be appreciated by those skilled in the art that there is no requirement that other portions of the effector domain (or other domains of the molecule) comprise natural sequences - in particular it may be desirable to combine the sequence modifications disclosed

20   herein with others, for instance selected from the literature, provided only that the required activities are retained. The skilled person will appreciate that binding molecules comprising such additionally-modified (e.g by way of amino acid addition, insertion, deletion

25   or substitution) effector domains fall within the scope of the present invention.

Particularly preferred may be 'null allotype' sequences, such as IgG heavy chain-derived sequences (see WO

30   92/16562) wherein allotypic residues are mutated to match those found in other human IgG subclass molecules. This may minimise the sequences being viewed as foreign by any individual.

The binding domain and target molecule

The peptide molecule comprises a binding domain capable of binding a target molecule.

The binding domain will have an ability to interact with a target molecule which will preferably be another polypeptide, but may be any target (e.g. carbohydrate, lipid (such as phospholipid) or nucleic acid). Preferably the interaction will be specific. The binding domain may derive from the same source or a different source to the effector domain.

For instance, while the effector domain will generally derive from an antibody, the binding domain may derive from any molecule with specificity for another molecule e.g. an enzyme, a hormone, a receptor (cell-bound or circulating) a cytokine or an antigen (which specifically binds an antibody).

Preferably, it comprises all or part of an antibody or a derivative thereof, particularly a natural or modified variable domain of an antibody. Thus a binding molecule according to the present invention may provide a rodent or *camelidae* (see WO 94/25591) originating antibody binding domain and a human immunoglobulin heavy chain as discussed above.

Also preferred may be molecules having more than one type of binding domain, such as bispecific antibodies (see e.g. PCT/US92/09965). In these cases one 'arm' binds to a target cell and the other binds to a second cell to trigger killing of the target. In such cases it may be

- 16 -

desirable to minimise the impact the effector portion, which might otherwise activate further cells which interfere with the desired outcome. The 'arms' themselves (i.e. the binding domain) may be based on Ig domains (e.g. Fab) or be from other proteins as in a fusion protein, as discussed in more detail below.

The binding molecule may comprise more than one polypeptide chain in association e.g. covalent or otherwise (e.g. hydrophobic interaction, ionic interaction, or linked via sulphide bridges). For instance it may comprise a light chain in conjunction with a heavy chain comprises the effector domain. Any appropriate light chain may be used e.g. the most common kappa light chain allotype is Km(3) in the general population. Therefore it may be desirable to utilise this common kappa light chain allotype, as relatively few members of the population would see it as foreign.

Typically the target will be an antigen present on a cell, or a receptor with a soluble ligand for which the antibody competes.

This may be selected as being a therapeutic target, whereby it is desired to bind it with a molecule having the properties discussed above, for instance to compete with or displace undesirable antibodies from it. Alternatively it may be desirable *per se* to bind the target molecule, without causing cell mediated destruction, antibody triggered inflammation or complement lysis. Equally the effector domain may function primarily in mediating transport and/or improved serum half life - in such cases the binding domain and

- 17 -

target molecule may be any system which would benefit from these qualities.

A selection of applications wherein binding molecules of the present invention could be used as therapeutic antibodies having inert (in some respects) Fc regions are set out below:

1)   Competition with maternal IgG alloantibodies for antigenic epitope on blood cells of fetus/neonate

Alloimmune disorders of fetal blood cells have a common pathogenesis. There is synthesis of IgG alloantibodies by the mother to a paternally inherited antigen on fetal red cells, granulocytes or platelets. This is followed by transplacental transport of the alloantibody. In the fetus or neonate, there is destruction of antibody-coated fetal blood cells, which may lead to a clinically significant fall in circulating levels of the relevant cells. Therapeutic antibodies to the relevant epitope, but with an Fc which does not trigger destruction, could compete with maternal antibody for binding to fetal cells, thus inhibiting their destruction.

Antibodies to red cell alloantigens lead to haemolytic disease of the fetus and neonate

The most important red cell alloantigens are in the Rhesus and Kell blood group systems. The incidence of haemolytic disease due to the RhD antigen has fallen dramatically since the introduction of post-natal prophylaxis, but cases still occur due to maternal sensitisation during the first pregnancy. Other Rhesus

- 18 -

antigens (C,c,E,e) can also cause haemolytic disease, as can antibodies to the Kell (K1) antigen, which in addition impair erythopoiesis in the fetal bone marrow.

5   Current therapy for severely affected fetuses consists of regular intra-uterine transfusion of antigen negative red cells. Infusions of non-specific immunoglobulin have not been shown to be effective in this condition. Anaemia and hyperbilirubinaemia in the neonate may require exchange

10   transfusion and/or phototherapy.

Experiments using inert Fc constructs with RhD specificity (designated Fog-1) have demonstrated their failure to trigger effector mechanisms (monocyte

15   activation as detected by chemiluminescence and ADCC), and importantly have also been shown to inhibit chemiluminescence and ADCC triggered by human sera containing polyclonal anti-D. ADCC and chemiluminescence have previously been shown to predict red cell

20   destruction *in vivo*. Previously published work has also demonstrated the ability of Fog-1 to compete with the majority of human anti-D sera for epitopes on the RhD protein.

25   *Antibodies to platelet alloantigens lead to fetal and neonatal alloimmune thrombocytopenia*

The most relevant antigen is human platelet antigen (HPA)-1a. HPA-1a antibodies complicate 1 in 350 normal

30   pregnancies, and lead to severe thrombocytopenia in 1 in 1200 fetuses. The most severely affected cases result in intracranial haemorrhage or death. The current options for therapy are weekly transfusions of HPA-1a negative platelets (which carries a risk of fetal death of

35   0.5%/procedure), and high dose intravenous immunoglobulin

given to the mother, which has variable and unpredictable efficacy. HPA-1a is defined by a single epitope on platelet glycoprotein IIIa (GPIIIa), and a single chain Fv recognising this epitope is available within the University of Cambridge Division of Transfusion Medicine (Griffin HM, Ouwehand WH. A human monoclonal antibody specific for the Leucine-33 (PA^{A1}, HPA-1a) form of platelet glycoprotein IIIa from a V gene phage display library. Blood 1995; 86: 4430-4436). The binding of an antibody based on this construct to human platelets has been shown to be inhibited by human anti-HPA-1a-sera. The inhibition was most consistent for sera with the highest titre of specific antibodies, which were associated with the most severe disease. This indicates that the recombinant antibody and sera antibodies bind to the same epitope on platelets.

In the above applications, and those below, in addition to a competitive binding effect, the therapeutic antibodies of the present invention may also trigger a beneficial inhibitory effect through FcγRIIb.

2)   Competition with autoantibody for epitope on autoantigen

Autoantibody mediated blood cell destruction

Haemolytic anemia by warm type IgG autoantibodies and thrombocytopenia by autoantibodies have a common mechanism of blood cell destruction. In both, autoantibodies target a selected repertoire of autoantigens (Rh and K on red cells, and GPIIb/IIIa,GPIb/IX/V on platelets). The binding of the autoantibody shortens the life-span of the blood cell leading to anemia or thrombocytopenia, respectively. It is not unlikely that red cell and platelet autoantibodies target a limited number of B-cell epitopes on their respective autoantigens. Recombinant variable domain antibodies against these epitopes can be generated by V gene phage display technology. Therapeutic antibodies to the relevant epitopes, but with inert Fc, could compete with the patient's blood cell autoantibodies for binding

- 20 -

to the autoantigen, thus inhibiting the destruction of the blood cell.

Goodpasture's syndrome (anti-glomerular basement membrane [GBM] disease)

This is a major cause of rapidly progressive glomerulonephritis, leading to lung haemorrhage and end-stage renal failure in weeks or months from onset. Conventional therapy depends on dialysis in combination with intensive plasma exchange and immunosuppressive therapy, which in itself may be complicated by life-threatening opportunistic fungal and viral infections. There is overwhelming evidence that this disease is mediated by autoantibodies, and the autoantigen has been localised to type IV collagen, a major component of GBM. It has been shown that autoantibodies in GBM disease bind to the non-collagenous (NC1) domain of the $\alpha3(IV)$-chain. The gene encoding this sequence (COL4A3) has been cloned and sequenced. We hypothesise that the effect of harmful anti-GBM autoantibodies can be neutralised by a monoclonal IgG competitor molecule which targets the immundominant epitope on $\alpha3(IV)NC1$ and has, by design, been equipped with a biologically inactive Fc domain. We will develop a recombinant chimaeric IgG antibody which binds the immunodominant $\alpha3(IV)NC1$ epitope but that lacks the classic effector functions. We will be able to achieve this as the genes encoding the variable domains of the murine anti-$\alpha3(IV)NC1$ have been developed and characterised (Pusey CD et al, Lab Invest 1987, 56;23-31 and Ross CN et al, Lab Invest 1996, 74;1051-1059).

Once again, in addition to a competitive binding effect, the therapeutic antibodies of the present invention may also trigger a beneficial inhibitory effect through FcγRIIb.

3)   Allergy and Asthma

Allergies and asthma result from innappropriate immune responses to common environmental antigens such as

proteins from grass pollens, house dust mites and many other common antigen sources, an example being the Der P 1 protein of the house dust mite *Dermatophagoides pteronyssinus.* Affected individuals make high levels of immunoglobulins particularly of the IgE class. These IgE antibodies are able to bind to the high affinity Fc-epsilon RI receptor on Mast cells and on Eosinophils. Cross-linking of the receptor bound IgE by the allergen results in activation of the cells and degranulation. This releases a number of inflammatory mediators which can cause severe symptoms or even death as a result of an anaphylactic reaction. Two mechanisms of action of a blocking antibody could be envisaged. Firstly an IgG antibody with an inert Fc region could compete for the binding of allergen to IgE. This would prevent the cross-linking of IgE and hence prevent the activation of the cells. For this mechanism the IgG antibody with inert Fc would have to compete directly for the binding of the allergen with the IgE.

A second, significant, mechanism would involve the role of negative signalling through the FcγRIIb receptor. It has been shown that the cross-linking of Fc gamma RIIB and Fc epsilon RI results in an inhibition of the activation signals normally seen when only Fc epsilon RI receptors are cross-linked. Thus the introduction of an IgG antibody with an Fc binding capacity for Fc gamma RIIb and an antigen specificity for an allergen could result in a an inhibition of the activation of IgE coated Mast cells and Eosinophils. For this the IgG antibody would also mediate its strong negative affect if it bound the allergen by a different site to the IgE such that both could bind to the allergen at the same time.

4)   Inflammatory disorders *eg. Crohn's disease*

There are a number of disorders of the immune system which seem to cause pathology as a result of the chronic state of activation of immune cells (leukocytes), including T-lymphocytes, neutrophils and NK-cells. This chronic activation is normally seen as a state of inflammation with a continued migration of activated

cells into the tissues affected. In order to migrate into
the tissue the cells must receive and respond to
inflammatory mediators and then regulate adhesion
molecules to enable them to first adhere to the cells
5   lining the blood vessel walls and then to migrate
between the cells of the vessel walls and into the
tissue. It should be possible to stop this cycle of
inflammation by either blocking the adhesion molecules on
the surface of the leukocytes or the corresponding
10   ligands on the activated epithelial cells lining the
vessel walls. Such an activation antigen is VAP-1 and an
antibody with an inert Fc which binds to this molecule
should prevent leukocyte adherance and migration at sites
of the inflammation thus breaking the cycle of chronic
15   activation.

5)   Inhibition of ligand/receptor interaction

Sickle cell disease

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Homozygosity for the variant of human haemoglobin
characterised by a substitution of valine for glutamic
acid (HbSS) leads to chronic haemolysis and a tendency
for the molecule to undergo tactoid formation in the
25   deoxygenated state. This leads to the red cells adopting
a sickle shape in the microcirculation leading to sickle
'crises' in localised areas. These may be thrombotic (in
bone, lung, brain or abdomen), aplastic, haemolytic or
associated with massive red cell sequestration in spleen
30   and liver. It is postulated that during these crises red
cells adhere to endothelial cells. This process of
adhesion is based on the interaction of several receptor
with their respective ligands. Two of the dominant
adhesion pathways are the interaction between Lutheran
35   and laminin and between thrombospondin and an as yet
undefined red cell membrane lipid. In animal experiments
we have obtained evidence that recombinant human variable
domain antibodies against thrombospondin diminish the
adhesion of sickling red blood cells to endothelial
40   cells. We postulate that similar recombinant variable
domain antibodies against the laminin binding domain of
lutheran (the membrane proximal domain) which block the

interaction with laminin can be developed by V gene phage
display. These variable domain antibody fragments can be
equipped with inert Fc domains to produce therapeutic
antibodies able to interfere with the adherence of

5   sickling red blood cells to endothelial cells, without
causing red cell destruction.

Antibody mediated blocking of platelet collagen receptors

10   We have substantial evidence that two receptors are
crucial to platelet activation by subendothelial
collagens, an event initiating thrombosis; the integrin
$\alpha_2\beta_1$ (platelet glycoprotein Ia/IIa) which we view
primarily as adhesive in function, and the non-integrin

15   glycoprotein VI (GpVI) as essential for activation,
preceding secretion and aggregation. Recombinant human
antibodies may be generated by V gene phage display
recognising different domains within each receptor, and
these may be used to produce lead-antibodies with an

20   inert Fc domain for collagen-based anti-thrombotic
therapy. These may be used in the alleviation of
coronary thrombosis, of restenosis after angioplasty and
of thrombotic complications associated with bypass
grafting.

25

6)   Monoclonal antibodies are used sometimes to block
cell functions, eg OKT3 is used to immunosuppress T-cells
by blocking the T-cell receptor and CD18 antibodies are
used to prevent cell-cell adhesion through the integrin

30   molecules. However the binding of the Fc to Fc receptors
can trigger serious side effects through stimulating
cytokine release and inflammation.

7)   Antibody Fc regions are sometimes attached to other

35   recombinant proteins to give fusion molecules with
prolonged biological half-lives. Thus TNF receptor has
been attached to human IgG4 Fc to form a molecule which
inhibits the effects of soluble TNF, and CTLA4 has been
made as a fusion protein with IgG Fc and used to block

- 24 -

signalling through the B7 coreceptor (a ligand for CTLA4)
molecule on cell surfaces. However again cytokine
triggering by the Fc of the fusion protein is
undesirable.

5

V domains, or other binding regions, appropriate to the
types of application discussed above, where discussed
specifically, will be well known to those skilled in the
art. For instance a CD3 binding domain (e.g. YTH12.5) is
10   disclosed by Routledge et al (1991) Eur J Immunol 21,
2717-2725 and Bolt et al (1993) Eur J Immunol 23, 403-
411. A CD52 binding domain (e.g. CAMPATH-1) is disclosed
by Riechmann et al (1988) Nature 332, 323-327. A VAP-1
binding domain is disclosed by Salmi et al (1993) J Exp
15   Med 178:2250-60 and Smith et al (1998) J Exp Med 188: 17-
27. A Der p I domain (e.g. 2C7) is disclosed by McElveen
et al (1998) Clin Exp Allergy 28, 1427-1434.

Thus a binding molecule which did not bind to Fc
20   receptors and trigger killing, and did not activate
complement, but which did bind to a target molecule,
could be used in all of the above examples to minimise
any side effects. Specifically, such a 'blocking'
antibody could be introduced in situations 1-5 above and
25   prevent the undesirable destruction by the naturally
occurring antibodies. The same blocking type Fc regions
would be the Fc regions of choice to use for recombinant
antibodies such as the CD3 or CD18 antibodies in 6 above
or as the Fc for fusions in 7 above.

30

The binding and effector domains may be combined by any
suitable method. For instance domains may be linked
covalently through side chains. Alternatively,
sulphydryl groups generated by the chemical reduction of
35   cysteine residues have been used to cross-link antibody
domains (Rhind, S K (1990) EP 0385601 Cross-linked
antibodies and processes for their preparation).
Finally, chemical modification of carbohydrate groups has
been used to generate reactive groups for cross-linking
40   purposes. These methods are standard techniques

available to those skilled in the art.   They may be
particularly applicable in embodiments wherein the
binding polypeptide contains non-protein portions or
groups.

Generally it may be more appropriate to use recombinant
techniques to express the binding molecule in the form of
a fusion protein.   Methods and materials employing this
approach form further aspects of the present invention,
as set out below.

Nucleic acids

In one aspect of the present invention there is disclosed
a nucleic acid encoding a binding molecule as described
above.

Nucleic acid according to the present invention may
include cDNA, RNA, genomic DNA (including introns) and
modified nucleic acids or nucleic acid analogs (e.g.
peptide nucleic acid).   Where a DNA sequence is
specified, e.g. with reference to a Figure, unless
context requires otherwise the RNA equivalent, with U
substituted for T where it occurs, is encompassed.

Nucleic acid molecules according to the present invention
may be provided isolated and/or purified from their
natural environment, in substantially pure or homogeneous
form, or free or substantially free of other nucleic
acids of the species of origin.   Where used herein, the
term "isolated" encompasses all of these possibilities.

The nucleic acid molecules may be wholly or partially
synthetic.   In particular they may be recombinant in that
nucleic acid sequences which are not found together in
nature (do not run contiguously) have been ligated or
otherwise combined artificially.   Alternatively they may
have been synthesised directly e.g. using an automated
synthesiser.

In a further aspect there is disclosed a nucleic
construct, e.g. a replicable vector, comprising the
nucleic acid sequence.

5   A vector including nucleic acid according to the present
       invention need not include a promoter or other regulatory
       sequence, particularly if the vector is to be used to
       introduce the nucleic acid into cells for recombination
       into the genome.

10

       Preferably the nucleic acid in the vector is under the
       control of, and operably linked to, an appropriate
       promoter or other regulatory elements for transcription
       in a host cell such as a microbial, (e.g. bacterial,
15   yeast, filamentous fungal) or eucaryotic (e.g. insect,
       plant, mammalian) cell.

       Particularly, the vector may contain a gene (e.g. *gpt*) to
       allow selection in a host or of a host cell, and one or
20   more enhancers appropriate to the host.

       The vector may be a bi-functional expression vector which
       functions in multiple hosts. In the case of genomic DNA,
       this may contain its own promoter or other regulatory
25   elements and in the case of cDNA this may be under the
       control of an appropriate promoter or other regulatory
       elements for expression in the host cell.

       By "promoter" is meant a sequence of nucleotides from
30   which transcription may be initiated of DNA operably
       linked downstream (i.e. in the 3' direction on the sense
       strand of double-stranded DNA). The promoter may
       optionally be an inducible promoter.

35   "Operably linked" means joined as part of the same
       nucleic acid molecule, suitably positioned and oriented
       for transcription to be initiated from the promoter.
       DNA operably linked to a promoter is "under
       transcriptional initiation regulation" of the promoter.

40

Thus this aspect of the invention provides a gene construct, preferably a replicable vector, comprising a promoter operatively linked to a nucleotide sequence provided by the present invention.

Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al,* 1989, Cold Spring Harbor Laboratory Press.

Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology,* Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

Host cells & methods

Also embraced by the present invention are cells transformed by expression vectors defined above. Also provided are cell cultures (preferably rodent) and products of cell cultures containing the binding molecules.

Also provided are methods of making binding molecules according to the present invention comprising:
(i) combining a nucleic acid encoding a binding domain with a nucleic acid encoding an effector domain to form a nucleic acid construct;
(ii) causing or allowing the expression of the construct in a suitable host cell.

Combination, to produce a construct, can be by any convenient method known to those skilled in the art, for instance by ligation of fragments (e.g. restriction fragments) or using different templates in one or more
5   amplification steps e.g. using PCR.

Methods of producing antibodies (and hence binding domains) include immunising a mammal (e.g. human, mouse, rat, rabbit, horse, goat, sheep, camel or monkey) with a
10   suitable target protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and might be screened, preferably using binding of antibody to antigen of interest.
15

For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, 1992, Nature 357: 80-82).

20   Cloning and expression of Chimaeric antibodies is described in EP-A-0120694 and EP-A-0125023.

The nucleic acid encoding the effector domain can be generated, in the light of the present disclosure, by
25   site directed mutagenesis, for instance by methods disclosed herein or in the published art (see e.g. WO 92/16562 or WO 95/05468 both of Lynxvale Ltd).

Other aspects
30

Also provided is use of the binding molecules of the present invention to prevent, inhibit, or otherwise interfere with the binding of a second binding molecule to a target molecule. This may involve competing with,
35   or displacing, an antibody from a therapeutically relevant target antigen or cell.

The present invention also provides a reagent which comprises a binding molecule as above, whether produced
40   recombinantly or otherwise.

The present invention also provides a pharmaceutical preparation which comprises a binding molecule as above, plus a pharmaceutically acceptable carrier.

The present invention also provides a method of treating a patient which comprises administering a pharmaceutical preparation as above to the patient, or to a sample (e.g. a blood sample) removed from that patient, which is subsequently returned to the patient. Particularly a method of treatment for the following diseases: Graft-vs-host disease; host-vs-graft disease; organ transplant rejection; bone-marrow transplant rejection; autoimmunity; alloimmunity; allergy; chronic or acute inflammatory diseases.

The present invention also provides a method of treating a patient which comprises causing or allowing the expression of a nucleic acid encoding a binding molecule as described above, whereby the binding molecule exerts its effects *in vivo* in the patient. Generally the expression will occur in the patient, or in certain specialised circumstances where the patient is an unborn infant, in the mother of the patient.

Also provided is the use of a binding molecule as above in the preparation of a pharmaceutical to modify an immune response, particularly a pharmaceutical for the treatment of the diseases discussed above.

In order that the present invention is more fully understood embodiments will now be described in more detail, by way of example only, and not by way of limitation. Other embodiments falling within the scope of the invention may occur to those skilled in the art in the light of these.

FIGURES

Figure 1

Rosetting of FcγRI-bearing cells by RBC coated with Fog-1

antibodies. R_2R_2 RBC were coated with Fog-1 antibodies at
a range of antibody concentrations, incubated with B2KA
cells growing in a 96-well plate and the percentage of
B2KA cells with rosettes of RBC determined. Error bars
5   indicate the standard deviation values for triplicate
wells. For the mutants Fog-1 G1Δb, G1Δc, G1Δab, G1Δac,
G2Δa, G4Δb and G4Δc, as for G2 (shown), there was no
rosetting between B2KA cells and RBC at any of the
coating concentrations.

10

Figure 2
Fluorescent staining of FcγRI-bearing cells. FcγRI
transfectant cell lines, B2KA(a and b) and 3T3+FcγRI+γ-
chain (c and d) were incubated sequentially with
15   antibodies of the CAMPATH-1 (a and c) or Fog-1 (b and d)
series, biotinylated anti-human κ antibodies and
ExtrAvidin-FITC. The fluorescence intensities were
measured for 10000 events and the geometric mean channel
of fluorescence plotted.

20

Figure 3
Histogram representation of fluorescently stained FcγRI-
bearing cells. B2KA cells were stained as in Figure 2
using 100 μg/ml antibodies from the CAMPATH-1 series.
25   The histogram plots showing the number of cells falling
in each fluorescence channel were overlaid for
representative antibodies.

Figure 4
30   CL response of human monocytes to RBC sensitized with
Fog-1 series of antibodies. R_1R_1 RBC were coated with
antibodies over a range of concentrations. The number of
antibody molecules bound per cell and the CL response of
moncytes to the RBC was determined for each sample as
35   described.

Figure 5
Inhibition of CL due to Fog-1 G1 by other Fog-1
antibodies. RBC were sensitized with 2 μg/ml Fog-1 G1
40   and different concentrations of the Fog-1 Ab indicated.

These Ab gave a low CL response in Figure 4. The CL
response of monocytes was measured. The response due to
2 µg/ml G1 alone is taken as 100%.

5   Figure 6
Inhibition of CL response to clinical sera by Fog-1 G2Δa.
RBC were sensitized with a constant amount of Fog-1 G1
(20 µg/ml) or clinically relevant sera and different
amounts of Fog-1 G2Δa. 100% response was achieved with a
10   standard amount of BRAD 5. In the absence of Fog-1 G2Δa,
the % responses were G1: 150%, sera A: 142%, sera B:
265%, sera C: 200%, sera D: 163%, sera E: 94%, anti-C+D
sera: 259% and anti-K sera: 119%.

15   Figure 7
Complement lysis mediated by CAMPATH-1 series of
antibodies. Human PBMC were labelled with ^{51}Cr and
incubated with the antibodies in the presence of serum as
a source of complement. The % specific Cr release is
20   plotted as a measure of lysis occurring.

Figure 8
Inhibition by CAMPATH-1 G2Δa of complement lysis mediated
by CAMPATH-1 G1. Complement lysis was carried out as in
25   Figure 7 but the samples contained a constant amount
(6.25 µg/ml final concentration) of CAMPATH-1 G1 and
increasing quantities of CAMPATH-1 G2Δa.

Figure 9
30   ADCC mediated by CAMPATH-1 series of antibodies. Human
PBMC were labelled with ^{51}Cr and incubated with antibody.
After washing, the cells were incubated with further
PBMC, acting as effector cells, in an effector:target
ratio of 20:1. The % specific Cr release is plotted as a
35   measure of lysis occurring.

Figure 10a
ADCC of RhD$^+$ RBC mediated by Fog-1 series of antibodies

40   Figure 10b

- 32 -

ADCC of RhD⁺ RBC mediated by Fog-1 series of antibodies

Figure 11a
Inhibition by Fog-1 antibodies of the ADCC of RhD⁺ RBC
5   mediated by Fog-1 G1 at 2 ng/mg

Figure 11b
Inhibition by Fog-1 antibodies of the ADCC of RhD⁺ RBC
mediated by Fog-1 G1. RBC were sensitized in a mixture
10   of antibodies consisting of a constant amount of Fog-1 G1
(2 ng/ml) and different concentrations of the inhibitor
antibodies.

Figure 12
15   Inhibition by Fog-1 antibodies of the ADCC of RhD⁺ RBC
mediated by polyclonal anti-RhD at 3 ng/mg

Figure 13a
Fluorescent staining of FcγRIIa 131H/H-bearing cells.
20   Cells of the transfectant line 3T6+FcγRIIa 131H/H were
incubated with the Fog-1 antibodies complexed with goat
F(ab')₂ anti-human κ and then with FITC-conjugated donkey
anti-goat IgG. The fluorescence intensities were
measured for 10000 events and the geometric mean channel
25   of fluorescence plotted.

Figure 13b
Fluorescent staining of FcγRIIa 131R/R-bearing cells.
Cells of the transfectant line 3T6+FcγRIIa 131R/R were
30   incubated with the Fog-1 antibodies complexed with FITC-
conjugated goat F(ab')₂ anti-human κ. The fluorescence
intensities were measured for 10000 events and the
geometric mean channel of fluorescence plotted.

35   Figure 14a
Fluorescent staining of FcγRIIb1*-bearing cells. The
experiment was carried out as in Figure 13b using the
transfectant line 3T6+FcγRIIb1* and complexing the Fog-1
antibodies using a mixture of FITC-conjugated and
40   unlabelled goat F(ab')₂ anti-human κ.

Figure 14b

Fluorescent staining of FcγRIIIb NA1-bearing cells. The
experiment was carried out as in Figure 13 using the
transfectant line CHO + FcγRIIIb NA1.

Figure 14c

Fluorescent staining of FcγRIIIb NA2-bearing cells. The
experiment was carried out as in Figure 13 using the
transfectant line CHO + FcγRIIIb NA2.

Figure 15

This shows Table 1, which compares the mutations made to
wildtype G1, G2 and G4 sequences.

Figure 16

This shows Table 2, which is a summary of antibody
activities.

~~Figure 17~~

~~This shows the Sequences of certain modified and wild-
type C$_H$2 sequences, including those designated G1Δab,
G2Δa, G1Δac.~~

EXAMPLES

General Materials and Methods

Construction of expression vectors

The starting point for the IgG1 constant region was the
human IgG1 constant region gene of allotype G1m(1,17) in
a version of the vector M13tg131 which contains a
modified polylinker (Clark, M. R.:WO 92/16562). The
2.3kb IgG1 insert thus has a BamHI site at the 5' end and
contains a HindIII site adjacent to the BamHI site. At
the 3' end, downstream of the polyadenylation signal, the
following sites occur in the order 5' to 3': SphI, NotI,
BglII, BamHI. The human IgG2 constant region gene had
been obtained as a HindIII-SphI fragment in M13tg131 and
the HindIII site had been destroyed by digesting with

HindIII, filling in the overhanging ends and ligating the ends together again. The SalI-SphI fragment of this vector was cloned to replace the equivalent fragment in the IgG1 vector described above. The human IgG4 constant

5   region gene had been obtained as a HindIII-SmaI fragment in M13tg131 and the HindIII site destroyed. The SmaI site occurs between the 3' end of the CH3 exon and the polyadenylation site so the polyadenylation site was restored by adding the SmaI fragment from the IgG1

10   vector, which comprises DNA from between the equivalent SmaI site in the IgG1 gene and the SmaI site downstream of the gene in the polylinker.

The first procedure was to introduce an XbaI restriction

15   site between the CH1 and hinge exons, a XhoI site between the hinge and CH2 exons and a KpnI site between the CH2 and CH3 exons in order to facilitate exchange of mutant exon sequences. This was similar to the manipulation of IgG1 and IgG4 genes carried out previously (Greenwood,

20   J., Clark, M. and Waldmann, H. (1993) Structural motifs involved in human IgG antibody effector functions. Eur. J. Immunol. 23, 1098-1104)

To provide the template DNAs, E. coli RZ1032 was

25   infected with the M13 described above and ssDNA prepared. The strain is dut⁻ung⁻ so the ssDNA produced should contain some uridine in place of thymidine.

The oligonucleotides used to introduce the mutations

30   were:
between the hinge and CH2 exons
MO10   5' GGA TGC AGG CTA CTC GAG GGC ACC TG 3'
between the CH2 and CH3 exons
MO11   5' TGT CCA TGT GGC CCT GGT ACC CCA CGG GT 3'

35   between the CH1 and hinge exons
MO12   5' GAG CCT GCT TCC TCT AGA CAC CCT CCC T 3'
Restriction sites are underlined.

The oligonucleotides were phosphorylated in 50 μl

40   reactions containing 25 pmol oligonucleotide and 5u T4

- 35 -

polynucleotide kinase (nbl) in 70 mM Tris HCl pH7.6, 10
mM MgCl$_2$, 100 mM KCl, 5 mM DTT, 0.5 mg/ml BSA, 1 mM ATP.
Reactions were incubated at 37C for 1h and heated at 70C
for 5 min.

5

To anneal the mutagenic oligonucleotides to the template
DNA, 500 ng uridine-containing DNA and 1 pmol each
phosphorylated oligonucleotide were incubated in 20 µl
of 40 mM Tris HCl pH7.5, 20 mM MgCl$_2$, 50 mM NaCl at 80C
10 for 5 min and allowed to cool slowly to 37C. The volume
was increased to 30 µl with the same buffer and DTT added
to 7 mM, ATP to 1 mM and dATP, dCTP, dGTP and dTTP each
to 250 µM. 5 u T7 DNA polymerase (unmodified, United
States Biochemical) and 0.5 u T4 DNA ligase (Gibco BRL)
15 were added and the reaction incubated at room temperature
for 16 h to synthesise the mutant strand. The DNA was
ethanol precipitated, dissolved 50 µl of 20 mM Tris HCl
pH8.0, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA and 1 u uracil
DNA glycosylase (New England Biolabs) added. After
20 incubating at 37C for 2 h, 50 µl 400 mM NaOH was added
and the reaction left at room temperature for 5 min to
fragment the template strand of DNA. The DNA was ethanol
precipitated, dissolved in H$_2$O and transformed into *E.
coli* TG1. Replicative form (RF) DNA was made for a
25 selection of the resultant M13 clones and digested to
find clones which contained the required *Xba*I, *Xho*I and
*Kpn*I restriction sites. Suitable clones were obtained
for the IgG1 and 4 vectors but MO12 appeared to be
misannealing in the IgG2 vector so the mutagenesis was
30 repeated for IgG2 without this oligonucleotide as the
site between the CH1 and hinge exons was not necessary
for these experiments. For each vector, the DNA
sequences of the exons were confirmed by sequencing.

35 ~~The changes in CH2 at amino acid positions 327, 330 and~~
331 (Δa mutation) were to be introduced using the
oligonucleotides:
MO22BACK (coding strand):
5' TCT CCA ACA AAG GCC TCC CGT CCT CCA TCG AGA AAA 3'
40 ~~MO22 (complementary strand):~~

5' TTT TCT CGA TGG AGG ACG GGA GGC CTT TGT TGG AGA 3'

The changes in CH2 at positions 233 to 236 (Δb and Δc mutation) were to be introduced using the oligonucleotides:-

MO7BACK (coding strand and encoding Δc mutation):

5' TCC TCA GCA CCT CCA GTC GCG GGG GGA CCG TCA GTC 3'

MO21 (complementary strand and encoding Δb mutation):

5' GAC TGA CGG TCC CGC GAC TGG AGG TGC TGA GGA 3'

The mutations were to be introduced by overlap extension PCR which also required the oligonucleotides MO11 and MO10BACK:

5' CAG GTG CCC TCG AGT AGC CTG CAT CC 3'

XhoI restriction site is underlined.

For the Δa mutation, the first set of PCRs used IgG1 and IgG2 templates amplified with MO22 and MO10BACK and with MO22BACK and MO11. For the Δb and Δc mutations, the first set of PCRs used IgG1 and IgG4 templates with MO21 and MO10BACK and with MO7BACK and MO11. In the final product, DNAs originating from a strand primed with MO21 would have the Δb mutation and those originating from MO22BACK would carry the Δc mutation. Each PCR contained about 30 ng M13tg131+constant region ssDNA, 25 pmol each oligonucleotide and 1 u Pwo DNA polymerase (Boehringer Mannheim) in 50 ul of 10 mM Tris HCl, pH8.85, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ and 250 μM each dATG, dCTP, dGTP and dTTP. The reactions were subjected to 14 cycles of 94C, 30 s; 50C, 30 s; 72C, 60 s, followed by 72C, 5 min to end. Bands representing product DNAs of the expected sizes were excised from low melting point agarose and melted in 100 μl H_2O. For each mutation, the two initial PCR products were joined together by overlap extension PCR. About 4 μl total of the melted gel slices, such that the initial PCR products were in equimolar amounts, were mixed with 25 pmol each MO10BACK and MO11 and other components as above. The PCR was carried out over 18 cycles as above except that the annealing temperature was reduced from 50C to 48C. The products obtained, which contained the entire CH2 exon, were purified and digested with XhoI and KpnI. The RF DNAs of the mutated

M13tg131+constant region vectors, containing the extra
restriction sites as described above, were digested with
XhoI and *KpnI* to remove the existing CH2 DNAs and the
mutant CH2 regions ligated in. The DNA samples were
5   transformed into *E. coli* TG1. DNA of representative
clones was sequenced to identify correctly mutated
constant regions.

In order to obtain IgG1 vectors with both Δa and Δb or
10   Δc, DNA, representing a Δa mutant, was used as the
template for a second round of PCRs to introduce the Δb
and Δc mutations as described above.

The IgG1, 2 and 4 wild type and mutated constant region
15   genes were each excised from RF DNA as a *Bam*HI - *Not*I
fragment and cloned into the modified CAMPATH Hu4VH
HuIgG1 pSVgpt vector (Clark, M. R.: Lynxvale Binding
Molecules as above) to replace the existing constant
region. The resulting vectors were designated
20   pSVgptCAMPATHHu4VHHuIgG1Δa, etc. The vector also contains
the *gpt* gene to allow selection in mammalian cells, the
murine immunoglobulin heavy chain enhancer and the
CAMPATH-1 Hu4VH variable region DNA so that it carries a
complete heavy chain gene which can be expressed in
25   mammalian cells. The CAMPATH-1 humanised light chain
gene exists in the expression vector CAMPATH HuVL pSVneo
(Reichmann, L., Clark, M. R., Waldmann, H. and Winter, G.
(1988) Nature 332, 323-327).

30   The Fog1 variable region DNAs (Bye, J. M., Carter, C.,
Cui, Y., Gorick, B. D., Songsivilai, S., Winter, G.,
Hughes-Jones, N. C. and Marks, J. D. (1992) Germline
variable region gene segment derivation of human
monoclonal anti-Rh(D) antibodies. J. Clin. Invest. 90,
35   2481-2490) were obtained in the vector pHEN1. They were
amplified by PCR using the oligonucleotides:-
FOG1VHBACK   5' TCC ACA GGT GTC CAC TCC CAG GTG CAT CTA
                       CAG CAG 3'

FOG1VHFOR   5' GAG GTT GTA AGG ACT CAC CTG AGG AGA CGG
40   TGA CCG T 3'

- 38 -

FOG1VKBACK 5' TCC ACA GGT GTC CAC TCC GAC ATC CAG ATG
ACC CAG 3'

FOG1VKFOR 5' GAG GTT GTA AGG ACT CAC GTT TGA TCT CCA
GCT TGG T 3'

5   The 5' portion of the insert in the vector M13VHPCR1
(Orlandi, R., Gussow, D. H., Jones, P. T. and Winter, G.
(1989) Proc. Natl. Acad. Sci. USA 86, 3833), comprising
the promoter and DNA encoding the signal peptide was
amplified using the universal M13 reverse primer and VO3:

10   5' GGA GTG GAC ACC TGT GGA GA 3'
DNA, 3' of the V_H in M13VHPCR1 and representing the 5' end
of the V_H-C_H intron, was obtained by PCR using the
universal M13 -40 primer and VO4:
5' GTG AGT CCT TAC AAC CTC TC 3'

15   These two segments of DNA were joined sequentially to
both the Fog-1 V_H and Fog-1 V_κ amplified DNA by overlap
extension PCR as described above. The BamHI restriction
site internal to the Fog-1 V_H was deleted by the same
method using oligonucleotides which removed the

20   recognition site without changing the amino acids
encoded. The complete PCR products were cloned into
M13mp19 as HindIII - BamHI fragments and their DNA
sequences confirmed.

25   The HindIII - BamHI fragment containing the Fog-1 V_H was
used to replace the fragment containing the CAMPATH-1 V_H
in the pSVgpt vectors described above, giving expression
vectors designated pSVgptFog1VHHuIgG2, etc. For the IgG1
vectors, the extra HindIII restriction site at the 5' end

30   of the constant region DNAs meant that it was not
possible to simply exchange the HindIII - BamHI variable
region fragment. Instead, the relevant
pSVgptCAMPATHHu4VHHuIgG1 vectors were digested with
HindIII. Linkers, designed to delete the HindIII site

35   and add a BamHI site, were ligated onto the cut ends.
The DNAs were then digested with BamHI and NotI so that
the constant regions could be isolated and these were
cloned into pSVgptFog1VHHuIgG2 to replace the IgG2
constant region.

40

- 39 -

The *Hind*III - *Bam*HI fragment containing the Fog-1 V_κ was transferred to the vector pSV*hyg*-HuCK (Orlandi *et al.*, 1989) which already contains the murine immunoglobulin heavy chain enhancer and the human κ constant region
5   gene. The resulting expression vector was called pSVhygFog1VKHuCK.

Production of antibodies

10   10 μg of each heavy chain expression vector and 20 μg of the relevant light chain expression vector were linearised by digestion with *Pvu*I and combined in 50 μl of H_2O. Cells of the non-secreting rat myeloma line, YB2/0, were grown to semi-confluency in Iscove's modified
15   Dulbecco's medium (IMDM) with 5% foetal bovine serum (FBS). 10^7 cells were collected by centrifugation, resuspended in 0.5 ml medium and transferred to a GenePulser cuvette (BioRad). The DNA was added and the mixture incubated on ice for 5 min. The cells were given
20   one pulse of 960 μF/170 V and returned to ice for 15 min before being placed in a flask in 20 ml IMDM + 10% FBS. They were incubated at 37C, 5% CO_2 in a humidified atmosphere. After 24 h, the volume was doubled and the medium made selective by addition of mycophenolic acid to
25   0.8 μg/ml and xanthine to 250 μg/ml. The cells were aliquotted over two 96-well plates. About 18 d after selection was applied, colonies were visible and the supernatants were assayed for the presence of IgG by ELISA. Briefly, microtitre-plate wells were coated with
30   goat anti-human IgG, Fc-specific antibodies (Sigma) and then incubated with 5-fold dilutions of the supernatants. Bound antibody was detected by incubating with HRPO-conjugated goat anti-human κ antibodies (Seralab) and developing the assay with *o*-phenylenediamine substrate.
35   Cells from wells containing the highest amounts of antibody were expanded and stocks cryopreserved.

The cell line secreting the highest amounts of Ab was expanded to 500 ml in IMDM + 2% FBS to provide saturated
40   supernatant for antibody purification. The supernatant

was cleared by centrifugation and made 0.1 M Tris HCl
pH8.0. Protein A-agarose (Sigma) was added and the
mixture stirred at 4C for 16 h. The agarose beads were
collected into a column and washed with 0.1 M Tris HCl
5   pH8.0, followed by 10 mM Tris HCl pH8.0. The antibody
was eluted with 1 ml aliquots of 0.1 M glycine pH3.0 into
100 μl samples of 1 M Tris HCl pH8.0 and the fractions
containing significant amounts of protein were identified
from A_{280nm} readings. These fractions were dialysed
10   against PBS, filter-sterilised and the A_{280nm} remeasured to
give the approximate antibody concentration
(concentration=A_{280nm} x 0.714 mg/ml).

The purity and integrity of the antibodies were
15   established by reducing SDS-PAGE, using 12.5% acrylamide.
The concentrations were checked in an ELISA which used
goat anti-human κ antibodies (Seralab) as the capture
reagent and biotinylated goat anti-human κ antibodies
(Sigma) followed by ExtrAvidin-HRPO (Sigma) for
20   detection. This meant that the nature of the heavy chain
was unlikely to influence the level of binding obtained.

Rosetting of FcγRI transfectants

25   Washed R_2R_2 RBC were incubated with Ab samples in 100 ml
PBS in 96-well plates at room temperature for 1 h. The
RBC were washed three times, resuspended in PBS and
incubated at 37C for 40 min with transfectants expressing
FcγRI cDNA, B2KA (S. Gorman and G. Hale, unpublished),
30   growing in 96-well plates. The supernatant was discarded
and the wells washed once to remove excess RBC. For each
well, 200 B2KA cells were examined and the number with
RBC rosettes noted. The mean percentage and standard
deviation for triplicate wells was plotted.
35   Alternatively, the sensitized RBC and B2KA cells were
mixed in microcentrifuge tubes, pelleted and gently
resuspended before transfer to a microscope slide.

Fluorescent staining of FcγR transfectants

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- 41 -

Transfectants expressing FcγRI cDNA, B2KA and
3T3+FcγRIa+γ-chain (van Urgt, M. J., Heijnen, I. A. F.
M., Capel, P. J. A., Park, S. Y., Ra, C., Saito, T.,
Verbeek, J. S. and van de Winkel, J. G. J. (1996) FcR γ-
chain is essential for both surface expression and
function of human FcγRI (CD64) *in vivo*. Blood 87, 3593-
3599), were obtained as single cell suspensions in
phosphate-buffered saline containing 0.1% (w/v) NaN$_3$, 0.1%
(w/v) BSA (wash buffer) following treatment with cell
dissociation buffer (Gibco BRL). Cells were pelleted at
10^5 cells/well in 96-well plates, resuspended in 100 μl
dilutions of the CAMPATH-1 or Fog-1 Ab and incubated on
ice for 30 min. Cells were washed three times 150
μl/well wash buffer and similarly incubated with 20 μg/ml
biotin-conjugated goat anti-human κ-chain Ab (Sigma) and
then with 20 μg/ml ExtrAvidin-FITC (Sigma). After the
final wash, cells were fixed in 100 μl wash buffer
containing 1%(v/v) formaldehyde. Surface expression of
FcγRI was confirmed by staining with CD64 mAb (Serotec)
and FITC-conjugated goat and mouse IgG Ab (Sigma).
Fluorescence intensities were measured on a FACScan
(Becton Dickinson).

For transfectants bearing FcγRII, 3T6 + FcγRIIa 131H/H,
3T6 + FcγRIIa 131R/R (Warmerdam, P. A. M., van de Winkel,
J. G. J., Gosselin, E. J., and Capel, P. J. A. (1990)
Molecular basis for a polymorphism of human Fcγ receptor
II (CD32). J. Exp. Med. 172, 19-25; Warmerdam, P. A. M.,
van de Winkel, J. G. J., Vlug, A., Westerdaal, N. A. C.
and Capel, P. J. A. (1991) A single amino acid in the
second Ig-like domain of the human Fcγ receptor II is
critical for human IgG2 binding. J. Immunol. 147, 1338-
1343) and 3T6 + FcγRIIb1* (Warmerdam, P. A. M., van den
Herik-Oudijk, I. E., Parren, P. W. H. I., Westerdaal, N.
A. C., van de Winkel, J. G. J. and Capel, P. J. A.(1993)
Int. Immunol. 5, 239-247) the antibodies were complexed
before being incubated with the cells. For FcγRIIa
131H/H, the antibodies were mixed with equimolar amounts
of goat F(ab')$_2$ anti-human κ (Seralab) and incubated at
37C for 1 h. The complexes were then mixed with the

- 42 -

cells and the assay continued as above except that the detecting antibody was FITC-conjugated donkey anti-goat IgG (Serotec). For FcγRIIa 131R/R, the complexes were made using equimolar amounts of FITC-conjugated goat F(ab')$_2$ anti-human κ (Seralab), and for FcγRIIb1*, the complexes were made using equimolar amounts of a 1:1 mixture of FITC-conjugated and unlabelled goat F(ab')$_2$ anti-human κ. Thus for these receptors only one incubation step was needed.

For transfectants bearing FcγRIIIb, CHO + FcγRIIIb NA1 or NA2 (Bux, J., Kissel, K., Hofmann, C. and Santoso, S. (1999) The use of allele-specific recombinant Fc gamma receptor IIIb antigens for the detection of granulocyte antibodies. Blood 93, 357-362), staining was carried out as described for 3T6 + FcγRIIa 131H/H cells above.

Red Cell Sensitization

Group O R_1R_1 RBC were washed in PBS and resuspended in RPMI + 10% FBS at a final concentration of 5% v/v. 10 μl of cells was added to 50 μl mAb or RPMI/FBS in V-bottom well plates and incubated for 60 min at 37C. In some experiments, the mAb were serially diluted in RPMI/FBS to achieve a range of red cell-bound IgG. In competition experiments, the red cells were sensitized in a mixture of 25 μl competing mAb and 25 μl of wild-type mAb or 25 μl serum containing alloantibodies. After sensitization, cells were washed 4 times with 200 μl volumes of PBS and resuspended in 50 μl RPMI/FBS (final concentration = 1% v/v). In all experiments, an aliquot of cells (E-IgG) was used in the chemiluminescence (CL) assay and an aliquot was assayed by flow cytometry to determine the level of red cell-bound IgG.

Chemiluminescence Assay

PBMC were isolated by density gradient centrifugation from EDTA-anticoagulated blood pooled from 6 normal donors. PBMC were washed 4 times with PBS containing 1%

- 43 -

globulin-free BSA and then resuspended at 2×10^6/ml in Hank's Balanced Salt Solution (HBSS) containing 25% RPMI and 2.5% FBS. Aliquots (100 μl) were dispensed into 96 flat-bottomed white opaque plates and incubated for 2 h

5 at 37C in a humidified atmosphere of 5% CO_2 in air. The plates were then placed in a luminometer (Anthos Lucy 1, Labtech International, Uckfield, UK) and 100 μl HBSS containing 4×10^{-4} M luminol (Sigma) and 20 μL E-IgG were added to each well. The CL response was then monitored

10 at 37C for 60 minutes.

Determination of Red Cell Bound IgG

25 μl aliquots of E-IgG were transferred to a V-bottom

15 well plate, washed once with PBS, centrifuged to a pellet and resuspended in 50 μl F(ab)$_2$ FITC-anti-IgG (diluted 1/30 in PBS/1% BSA). After 30 min at room temperature, the cells were washed once with 200 μl PBS/BSA and kept on ice until analysed by flow cytometry (EPICS XL-MCL,

20 Coulter Electronics, Luton, UK). The mean channel fluorescence was recorded.

Mean channel fluorescence was converted to IgG molecules/cell by use of a standard curve which was

25 prepared by adding 100 μl of 5% v/v R_1R_1 cells to 900 μl of serial 2 fold dilutions of human monoclonal IgG1 anti-D (BRAD-5). Sensitized red cells were washed 3 times with PBS/BSA and resuspended to 1% v/v in PBS/BSA. 25 μl aliquots were removed and analysed by flow cytometry as

30 described above. The remaining red cells were counted, centrifuged to a pellet, lysed in a buffer containing Triton X-100 and IgG in lysates was determined by ELISA as described by Kumpel (Kumpel, B.M. (1990). A simple non-isotopic method for the quantitation of red cell-

35 bound immunoglobulin. *Vox Sanguinis*, 59, 34-39). The number of IgG molecules bound per red cell was deduced from the IgG concentration and the number of red cells from which each lysate was prepared. A standard curve was then plotted comparing fluorescence intensity with

40 the number of IgG molecules bound per red cell.

- 44 -

Complement lysis mediated by CAMPATH-1 series of antibodies

100 ml venous blood from a healthy volunteer was
defibrinated and components separated by density gradient
centrifugation using Ficoll-Paque Plus (Pharmacia). The
serum and mononuclear cell layers were removed to fresh
tubes. The cells were diluted into Iscove's modified
Dulbecco's medium (IMDM) and collected by centrifugation.
The cells were washed twice in IMDM whilst being combined
into one pellet which was resuspended in 200 μl IMDM.
900 μCi sodium [^{51}Cr] chromate was added and the cells
incubated at 37C for 40 min. 10 ml IMDM was added and
the cells pelleted. The cells were washed twice and
resuspended in IMDM at approximately 6 x 10^6 cells/ml. 50
μl aliquots of labelled cells were added to antibody
samples in 50 μl IMDM in 96-well plate wells. 100 μl
retained serum diluted 1:1 with IMDM was added to each
well and the plates incubated at 37C for 1 h. The plates
were centrifuged and the supernatants were sampled and
the relative amounts of ^{51}Cr released were measured in a
γ-counter. The level of spontaneous release was obtained
from samples were no antibody was added and a measure of
the total amount of ^{51}Cr available for release was found
from similar samples taken after resuspending the cells.
The % specific ^{51}Cr release was calculated from the
formula:

$$\frac{(sample\ counts\ -\ spontaneous\ counts) \times 100}{(total\ counts\ -\ spontaneous\ counts)}$$

The means and standard deviations of the triplicate
samples were plotted.

For the inhibition of complement lysis, antibody samples
contained a constant amount (6.25 μg/ml final
concentration) of CAMPATH-1 G1 and increasing quantities
of CAMPATH-1 G2Δa.

ADCC mediated by CAMPATH-1 series of antibodies

Peripheral blood mononuclear cells were prepared as described above. After washing, the cells were resuspended in IMDM supplemented with 5% FBS and transferred to flask which had been coated with CD3 antibody. The cells were grown at 37C, 5% CO_2 for three days. 5% of the cells were labelled with ^{51}Cr for use as target cells, washed and resuspended at 6×10^5 cells/ml in IMDM + 5% FBS. 50 μl aliquots were added to wells of 96-well plates containing 50 μl samples of antibodies in IMDM + 5% FBS. The target cells and antibodies were incubated at 37C for 1 h, RBC added as carriers and the cells pelleted. The cells were washed twice in IMDM. The remaining mononuclear cells were collected by centrifugation and resuspended at 4×10^6 cells/ml in IMDM + 5% FBS and 150 μl added to each well resuspending the target cells in the process. This gives an effector:target ratio of 20:1. The cells were centrifuged gently and placed in a tissue culture incubator for 6 h. Supernatant was sampled and specific ^{51}Cr release determined as described above. The mean values of specific release for the duplicate samples was plotted against the final antibody concentrations.

Example 1 - Generation and basic characterisation of antibodies

The mutations chosen to eliminate the effector functions are shown in Table 1 (Fig 15). The Δa mutation made in IgG1 and IgG2 genes introduces the IgG4 residues at positions 327, 330 and 331. Similarly, the IgG2 residues at positions 233 - 236 were introduced into IgG1 and IgG4 but, since IgG2 has a deletion at 236 where the other subclasses have a glycine residue, the mutation was made omitting (Δb) or including (Δc) G236.

Vectors allowing expression of CAMPATH-1 or Fog-1 V_H DNA in conjunction with the wildtype or mutant constant region genes were cotransfected with the appropriate light chain expression vectors into rat myeloma cells.

Stable transfectants were isolated, expanded and Ab purified from the supernatant on protein A-agarose.

5 CAMPATH-1H was selected as it provides a good targeting system for studying complement and cell mediated lysis *in vitro*.

For the Fog-1 Ab, a precipitate formed after purification but, once this had been removed by filter-sterilisation,
10 no further precipitation was noticed. Ab concentrations were estimated from the absorbance at 280 nm and were adjusted where necessary following an ELISA which measures the relative amounts of κ-chain present. The Ab were subjected to reducing SDS-PAGE. Each sample showed
15 two bands with apparent molecular weights of approximately 25 and 55 kDa which represent the expected sizes of the light and heavy chains. There was no discernible difference in size between the heavy chains of each Ab series but both chains of the Fog-1 Ab
20 appeared to be slightly smaller than their CAMPATH-1 counterparts. The fact that the heavy chain within each series appeared to have the same apparent molecular weight indicates that the mutations did not cause any extensive differences in the glycosylation of the
25 proteins. For the Ab with CAMPATH-1 specificity, the yield after purification varied from 0.6 to 9 μg/ml supernatant whereas the yield of soluble Fog-1 Ab was between 3 and 20 μg/ml. There was no correlation in the ranking of the purification yields for the two series of
30 antibodies suggesting that none of the mutations affected the production of the Ab or their ability to bind protein A.

The specificities of the two series of Ab were then
35 tested. The CAMPATH-1 Ab were shown to compete with clinical grade CAMPATH-1H in the binding of the anti-CAMPATH-1 idiotype mAb, YID13.9. The Fog-1 Ab where able to agglutinate RhD⁺ RBC in the presence of anti-human IgG Ab as cross-linking reagents. Similarly, the IgG
40 subclasses of the Fog1 Ab were examined by coating RhD⁺

RBC with the different Ab and looking at the agglutination pattern using anti-G1m(a), anti-IgG2 or anti-IgG4 Ab as the cross-linking Ab. The result indicated that the antibodies were of the correct subclasses. The agglutination of RhD^+ RBC by Fog-1 IgG1 and anti-G1m(a), by Fog-1 IgG2 and anti-IgG2 and by Fog-1 IgG4 and anti-IgG4 was then carried out in the presence of excess Ab from the CAMPATH-1 series. The CAMPATH-1 Ab were able to inhibit the agglutination, by competing for the cross-linking reagent, only where they were of the same subclass as the Fog-1 Ab, thus verifying their subclasses.

Example 2 - FcγRI binding

RBC with approximately 30 000 RhD sites per cell (R_2R_2) were coated with each of the 11 Fog-1 Ab over a range of concentrations and added to human FcγRI-expressing transfectants, B2KA, growing in wells. After incubation, excess RBC were washed away and the percentage of B2KA cells rosetted by RBC was recorded (Figure 1). For G1 and G1Δa, where IgG4 residues are included at positions 327, 330 and 331, similar levels of rosetting were achieved, with half-maximal rosetting occurring when the RBC were coated with Ab at about 0.1 μg/ml, a concentration at which Fog-1 Ab would be expected to occupy approximately one-third of the RhD sites. Slightly higher concentrations of G4 were needed to obtain the same levels of rosetting. No rosettes were formed when using RBC coated with the G1 and G4 Ab containing the Δb and Δc mutations or the G2 Ab. In the experiment shown in Figure 1, the highest coating concentration tested was 10 mg/ml, predicted to correspond to approximately 90% occupancy of RhD sites. The experiment was repeated using coating concentrations of up to 80 mg/ml, essentially saturating the RhD sites, and still no rosettes were seen for G2 and the Ab containing the Db or Dc mutations and thus incorporating IgG2 residues in the lower hinge region. This indicates that, even when the RBC were coated with these Ab at the maximum density for this antigen, there was insufficient

IgG/FcγgRI interaction for rosette formation.

Centrifuging the sensitized RBC and B2KA cells together before observing rosettes on a microscope slide was found to give a higher proportion of rosettes than incubating the cells in wells so this method was used to investigate the inhibition of rosette formation. R_2R_2 RBC were coated with a mixture of 1 mg/ml Fog-1 G1 and different amounts of Fog-1 G2Da or Fog-1 G4Db before mixing with B2KA cells. When 1 μg/ml Fog-1 G1 was used alone, the coated RBC formed rosettes on 95% of the B2KA cells whereas sensitization in the presence of 64 mg/ml G2Δa or G4Δb completely abolished the rosetting (data not shown).

The binding of Ab from both series to two different cell lines, which express the FcγRI cDNA on their surface, was measured by fluorescent staining. Figure 2 shows representative experiments. The level of surface-expressed FcγRI, as detected using the CD64 Ab, was higher for the 3T3 transfectants than for the B2KA line and this reflects in the higher signals obtained when measuring binding via the Fc. For both series, the G1 and G1Δa Ab bound to the receptor with the same apparent affinity indicating that the mutations at positions 327, 330 and 331 did not significantly affect the interaction. The binding of G4 Ab was approximately three-fold lower than that of the G1 and G1Δa Ab. Little binding was seen for the G2 Ab or any of the other mutant Ab, suggesting that the Δb and Δc mutations in IgG1 and IgG4 were sufficient to reduce binding to FcγRI by at least 10^4-fold. Ab containing the Δc mutation, especially G1Δc, showed a small degree of binding to FcγRI at the highest concentrations tested if the level of fluorescence is compared to the background or to the equivalent Ab with the Δb mutation. If the fluorescence intensity histograms are overlaid, as seen in Figure 3 for the highest concentrations of CAMPATH-1 Ab and B2KA cells, the plots for G1 and G1Δa coincide. There is a clear difference between the histograms for the G1Δb and G1Δc Ab.

Example 3 - FcγRI triggering measured by
chemiluminescence

In order to measure functional activity through FcγRI/II,
the chemiluminescent (CL) response of monocytes to RBC
sensitized with Ab from the Fog-1 series was measured and
plotted in relation to the number of Ab molecules bound
per RBC (Figure 4). A difference between the G1 and G1Δa
Ab is seen with higher amounts of Ab but both are give
higher responses than the G4 Ab across the range of Ab
concentrations. Significant triggering is achieved by
the G1Δc Ab and, to a lesser extent, by G1Δac and G4Δc
but the other Ab do not give any response.

Ab, which were known to be deficient in the triggering of
FcγRI from the previous section, were mixed in increasing
concentrations with a constant amount of Fog-1 G1 and
used to sensitize RBC. The CL response to the RBC is
shown in Figure 5. By comparing the CL response to that
obtained when titering G1 alone, it appears that six of
the eight Ab inhibit the reaction to an extent which
predicted if it is assumed that the mutants displace the
active G1 from RBC in proportion to their relative
concentrations. For G2, the inhibitory effect is delayed
in that about three-fold more G2 is needed to give the
same amount of inhibition. G1Δc inhibits to
approximately the same extent as the other mutants except
that the response is not reduced to zero.

Two papers which have discussed the usefulness of
chemiluminescence in predicting the severity of in-vivo
pathology are Hadley (1995) Transfusion Medicine Reviews
9:302-313 and Hadley et al (1998) Br J Obstet Gynaecol
105: 231-234.

In these assays a result above 30% chemiluminescence
produced by the BRAD-5 monoclonal antibody control would
be predictive of in-vivo pathology in HDN. Thus those
antibodies which can block to levels below 30% should be
suitable for therapy.

One of the mutant Ab, Fog-1 G2Δa was tested for its ability to inhibit the CL response to sera containing clinically significant Ab. The sera contained anti-RhD Ab or antiC+D and, in the absence of inhibitor, gave CL
5   responses of greater than 30% on this scale which is indicative of severe haemolytic disease of the newborn and the need for intrauterine transfusions. The sera were mixed with different concentrations of G2Δa, the mixtures used to sensitise RBC and the responses of
10 monocytes measured (Figure 6). The addition of G2Δa Ab reduced the CL signals due to all five anti-RhD sera to below the 30% cut-off. The amount of Ab needed to achieve this varied from 16 - 260 μg/ml, the range presumably reflecting the differing amounts and
15 affinities of anti-RhD Ab in the serum. There are two control sera. The anti-K serum cannot be blocked at all by G2Δa as its reactivity is directed towards a different antigen on the RBC. Only part of the activity of the anti-C+D serum could be inhibited by G2Δa.

20

Example 4 - Activity in complement lysis

Figure 7 shows that all the mutations made to the G1 and G2 CAMPATH-1 antibodies dramatically reduced their
25 ability to mediate complement lysis. When the assay was carried out using a constant amount of G1 and different amounts of G2Δa (Figure 8), the G2Δa antibody was able to block the killing of PBMC by CAMPATH-1 G1.

30 Example 5 - Activity in ADCC

The ability to mediate ADCC was measured for the CAMPATH-1 antibodies using human PBMC as target cells (Figure 9) and for the Fog-1 antibodies using RhD⁺ RBC as target
35 cells (Figures 10 and 10b). Figure 9 shows mixed abilities of the CAMPATH-1 antibodies in ADCC, with some of the mutants having very low activities. Figures 10 and 10b show that the Fog-1 antibody mutants G1Δab, G1Δac, G2Δa, G4Δb and G4Δc were unable to support any
40 killing of the RBC. In Figure 10, some lysis of RBC

- 51 -

sensitized with G2 or G4 is seen but these antibodies
have no apparent activity in the assay of Figure 10b.
This demonstrates the observation that the degree of
lysis may be dependent on the donor of the effector cells
and may even vary when using effector cells taken from
the same donor at different times. However, for the
mutants listed above, no activity above background levels
has been seen although a range of effector cell donors
have been tested.

Some of the Fog-1 antibodies were used to try to inhibit
the ADCC of RhD$^+$ RBC by Fog-1 G1 (Figures 11 and 11b) and
by a clinical sample of anti-RhD serum (Figure 12). The
figures show that all of the antibodies tested were able
to inhibit ADCC when mixed with the active antibodies
prior to RBC sensitisation. The Fog-1 mutant antibodies
G1Δb, G1Δab, G1Δac, G4Δb and G4Δc were particularly
effective at blocking ADCC.

Example 6 - FcγRII binding

Figures 13, 13b and 14 show the binding of complexes of
antibodies from the Fog-1 series to cells bearing FcγRIIa
131H/H, FcγRIIa 131R/R and FcγRIIb1* respectively. It is
necessary to form antibody complexes when measuring
binding to these receptors due to their low affinity for
individual antibody molecules. FcγRIIa 131H/H is an
allotype of FcγRIIa to which IgG2 antibodies are
expected to bind strongly and, indeed, G1 and G2 show a
strong binding activity (Figure 13). Addition of the
mutations to these two antibodies appears to give a
stepwise reduction in the levels of binding and the G1Δc
and G1Δac antibodies have only background levels of
binding as exhibited by the G4 antibodies. Figure 13b
shows that the antibodies have different relative
activities when binding to the 131R allotype of FcγRIIa
but the mutations made to the wildtype G1 antibody again
decrease binding to the receptor. All of the antibodies
show significantly more binding to the inhibitory
receptor, FcγRIIb1*, than the negative control samples of

- 52 -

cross-linking F(ab')$_2$ alone or an aglycosyl IgG1 antibody
complexed with the F(ab')$_2$ (Figure 14). Although the
binding of most mutants is reduced relative to the
corresponding wildtype antibodies, some mutants show
binding within two-fold of that exhibited by the wildtype
G1 antibody.

Example 6b - FcgRIII binding

Figures 14b and 14c show the binding of complexes of
antibodies from the Fog-1 series to cells bearing
FcγRIIIb of the allotypes NA1 and NA2 respectively. For
both allotypes, binding is seen for the G1 antibody and,
to a lesser extent, the G1Δa and G1Δc antibodies. No
binding is observed for the other mutant antibodies since
they show similar levels of fluorescence to the negative
control samples of cross-linking F(ab')$_2$ alone or an
aglycosyl IgG1 antibody complexed with the F(ab')$_2$.

Example 7 - Production of the anti-HPA-1a antibodies

The V$_H$ and V$_λ$ of the anti-HPA-1a scFv (Griffin,H.M. and
Ouwehand, W.H. (1995) A human monoclonal antibody
specific for the leucine-33 form of the platelet
glycoprotein IIIa from a V gene phage display library.
Blood 86, 4430-4436) were amplified and each attached to
leader sequence from the vector M13VHPCR1 (Orlandi et
al., 1989) by overlap extension PCR as described
previously. DNA, 3' of the V$_H$ in M13VHPCR1 and
representing the 5' end of the V$_H$-C$_H$ intron, was similarly
joined to the leader/V$_H$DNA. The product was cloned as a
HindIII- BamHI fragment into IgG1 and IgG2 expression
vector to replace the existing variable region fragment
and to give the vectors pSVgptB2VHHuIgG1 and
pSVgptB2VHHuIgG2.

The leader/ Vλ DNA was joined in frame to the human λ
chain constant region DNA of the Kern⁻Oz⁻ allotype
(Rabbitts, T.H. Forster, H. and Matthews, J.G. 1983. Mol.
Biol. Med.1:11), taken from an existing expression vector
(Routledge, E.G., Lloyd, I, Gorman, S.D., Clark, M. and

Waldmann, H. 1991, *Eur. J. Immunol.* 21:2717). The whole
λ gene was cloned into M13 as a *Hind*III-*Bam*HI fragment
and the murine heavy chain enhancer from pSV*hyg*-HuCK
(Orlandi et al., 1989) added 5' of the gene using

5   adapters so that the whole insert could be transferred to
pSV2*neo* (Southern, P. J. and Berg. P. 1982. *J. Mol. Appl.
Genet.* 1:327) as a *Bam*HI fragment. The vector was
designated pSV*neo*B2VλHuCλ.

10   The expression vectors were transfected into the rat
myeloma cell line YB2/0, transfectants selected and
antibody purified as described before. These B2IgG1 and
B2IgG2 antibodies can be used as control antibodies.

15   Once the preferred null constant regions have been
selected, the B2 VH *Hind*III-*Bam*HI fragment can be
introduced into expression vectors carrying the
appropriate constant region genes, replacing the existing
variable region fragment. The heavy chain expression

20   vectors can then be co-transfected with pSV*neo*B2VλHuCλ
into myeloma cells and the antibodies purified for use.

Example 8 - Therapeutic use of binding molecule

25   A therapeutic molecule according to the present invention
may be used to treat pregnancies complicated by HPA-1a
alloimmunisation, for instance by intravenous
administration to the mother, thereby relying on
placental transfer (e.g. via the FcRn) to provide a

30   therapeutic dose to the fetus.

An alternative is direct administration to the fetus by
percutaneous umbilical vessel sampling. This procedure is
currently performed in FAIT to deliver transfusions of

35   compatible platelets. Because of the short survival of
transfused platelets, the procedure may have to be
repeated many times during the course of a pregnancy. It
is however hazardous, with a risk of fetal loss of
0.5%/procedure.

40

However, fetal administration of a therapeutic antibody
would have the advantage that a much lower dose is likely
to be required, and therefore a combined approach using
the molecules of the present invention in conjunction
5   with platelet transfusion may be considered as a first
step in therapy. This approach may reduce or eliminate
the need for further platelet transfusions before
delivery.

10   Summary

The activities of the antibodies are summarised in Table
2 (Figure 16). As can be seen, binding molecules have
been produced which have reduced ability to bind to
15   FcγRI, FcγRIIa 131H/H, FcγRIIa 131R/R, FcγRIIIb NA1 and
FcγRIIIb NA2; are unable to trigger monocyte
chemiluminescence; cannot mediate complement lysis and
are not active in ADCC. However, the binding molecules
retain binding to the inhibitory receptor, FcγRIIb.
20   Other mutations previously used to knock out effector
functions, such as removing the glycosylation site in the
CH2 domain to make aglycosyl antibodies, may also
eliminate binding to this receptor which may not be
desirable.
25
Selected mutants have been shown to be able to inhibit
completely the rosetting of FcγRI-bearing cells by Fog-1
G1; the response of monocytes to Fog-1 G1-sensitised
RBC; the response of monocytes to polyclonal anti-RhD-
30   sensitised RBC; the killing of PBMC by complement lysis
with CAMPATH-1 G1; the killing of RBC by ADCC with Fog-1
G1; the killing of RBC by ADCC with polyclonal anti-RhD
serum.

35   The results herein demonstrate that altering even a
single residue in an IgG CH2 domain to correspond to a
different subclass can lead to different activities.
Thus for the three pairs of Db and Dc mutants: G1Δb and
G1Δc, G1Δab and G1Δac, G4Δb and G4Δc. Within each pair,
40   the antibodies differ only by the absence (Δb) or

presence (Δc) of G236. However, for most of the functions measured here, the Δb and Δc antibodies have different activities. The Db mutants are more active in binding to FcγRIIa 131H/H whereas the Dc mutants are more

5   active in FcγRI binding, FcγRIIIb NA1 and NA2 binding, monocyte activation and ADCC. The region where the Δb and Δc mutations are made is known as the lower hinge or hinge link region and is likely to have an extended structure, connecting the hinge to the remainder of the

10   CH2 domain. Addition or deletion of a residue from this region presumably alters the alignment of the lower hinge residues relative to receptor interaction sites in the remainder of the CH2 domain.

15   However it should be stressed that the effect of mutations cannot always be predicted from wildtype antibody activities, but will depend on the novel context (based on 'mixed' subclasses of IgG) in which the mutation is present. One example is in the assay of

20   complement lysis where the activity of the IgG2 antibody is only about three-fold lower than that of IgG1 but introducing IgG2 residues into IgG1 (G1Δb and G1Δc) eliminates lysis. Similarly, IgG1 and IgG2 show equal binding to FcγRIIa 131H but G1Δb and G1Δc activities are

25   50- and 10- fold lower respectively. In the ADCC assays of Figure 9 and 10, IgG2 and IgG4 give similar, low but measurable levels of lysis. Substituting residues between IgG2 and IgG4, as well as into IgG1, reduces activity. These data suggest that the wildtype

30   antibodies of the different human IgG subclasses and, presumably, the mutant antibodies may use different residues in binding to other molecules to trigger activities.